

Location of antithrombin-binding regions in rat skin heparin proteoglycans

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Rat skin heparin proteoglycan labelled biosynthetically with ^{35}S was fractionated on a column of antithrombin–Sephacrose into fractions with varying degrees of affinity for antithrombin. These were treated with NaOH to release heparin chains (M_r 60 000–100 000) by β -elimination or incubated with serum to produce fragments of the same order of size as commercial heparin (M_r 5000–30 000), by endoglycosidase cleavage. Chains and fragments were then fractionated on antithrombin–Sephacrose. The various fractions were deaminated with HNO_2 at pH 1.5 followed by reduction with NaBH_4 . Approx 90% of the incorporated ^3H was associated with disaccharides. These were fractionated by high-performance ion-exchange chromatography. A unique minor component corresponding to the sequence glucuronosyl-*N*-sulphoglucosaminyl (3,6-di-*O*-sulphate) in the polysaccharide was found only in fractions with high affinity for antithrombin. The glucosamine residue linked to C-4 of this glucuronosyl unit was predominantly (or exclusively) *N*-sulphated rather than *N*-acetylated, pointing to a structural difference between the antithrombin-binding region of rat heparin and that of pig mucosal heparin. Calculations based on the distribution of the glucosaminyl 3-*O*-sulphate group showed that approximately two-thirds of the total antithrombin-binding regions present in the unfractionated material were accommodated by only 20% of the proteoglycan molecules, and by 10% of the polysaccharide chains. While most of the proteoglycan molecules thus lacked such regions (and hence affinity for antithrombin) a minor proportion of the polysaccharide chains contained on the average three binding regions per molecule. These findings support by direct chemical analysis an earlier proposal, based on anticoagulant activities of similar rat skin heparin fractions, that the distribution of antithrombin-binding sites in intact heparin proteoglycans is markedly non-random.

INTRODUCTION

The blood anticoagulant activity of heparin, a sulphated glycosaminoglycan, is due to the presence of a specific pentasaccharide sequence (Lindahl *et al.*, 1984), the distinguishing feature of which is a 3-*O*-sulphated glucosamine residue (Fig. 1). Molecules possessing this sequence bind with high affinity to antithrombin, and thereby dramatically accelerate the rate at which this proteinase inhibitor inactivates enzymes involved in the coagulation process (Björk & Lindahl, 1982). Commercial preparations of heparin, M_r 5000–25 000, isolated from pig intestinal mucosa or bovine lung are readily separated by affinity chromatography on immobilized antithrombin into distinct fractions with high or low affinity for the inhibitor. The high-affinity fractions, usually about one-third of the total products, account for essentially all of the anticoagulant activity (Lindahl *et al.*, 1979; Rosenberg & Lam, 1979) and the 3-*O*-sulphated glucosamine residues (Lindahl *et al.*, 1980) of the starting material.

Heparin is synthesized as a proteoglycan with unusual structural properties (Horner, 1971; Robinson *et al.*, 1978). The polysaccharide chains, M_r 60 000–100 000, are much more extended than are those of commercially available heparin preparations, and are attached to a

peptide core composed essentially of alternating serine and glycine residues. Studies of heparin metabolism in murine mastocytoma cells have shown that the newly synthesized chains are degraded by an endo- β -D-glucuronidase (Ögren & Lindahl, 1975, 1976) to fragments similar in size to the commercially available polysaccharide. These fragments are stored in cytoplasmic granules of the mast cell. The distribution in the intact chains of antithrombin-binding pentasaccharide regions and of cleavage sites for the endoglucuronidase will determine the antithrombin-binding properties of the fragments. The substrate specificity of the glucuronidase appears to be more exacting than that of similar endoglycosidases from other sources such as platelets (Thunberg *et al.*, 1982a).

In certain tissues, e.g. rat skin, the heparin proteoglycan escapes degradation and can therefore be isolated as the intact macromolecule (Horner, 1971). The distribution of antithrombin-binding regions in this structure was studied by Horner & Young (1982), who subjected intact proteoglycan, single polysaccharide chains, or polysaccharide fragments obtained by enzymic depolymerization, to affinity chromatography on immobilized antithrombin, and then determined the anticoagulant activities of the resulting fractions. They concluded that the binding regions were accumulated in an apparently

Abbreviations used: HexUA, unspecified hexuronic acid; GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GlcNAc, 2-deoxy-2-acetamido-D-glucose (*N*-acetyl-D-glucosamine); aMan_R, 2,5-anhydro-D-mannitol formed by reduction of terminal 2,5-anhydro-D-mannose residue with NaBH_4 ; -NSO₃, *N*-sulphate, sulphamino group; -OSO₃, *O*-sulphate, ester sulphate group. The locations of *O*-sulphate groups are indicated in parentheses. In the description of heparin fractions PG denotes proteoglycans; C, polysaccharide chains; F, polysaccharide fragments; HA, high affinity for antithrombin; LA, low affinity for antithrombin.

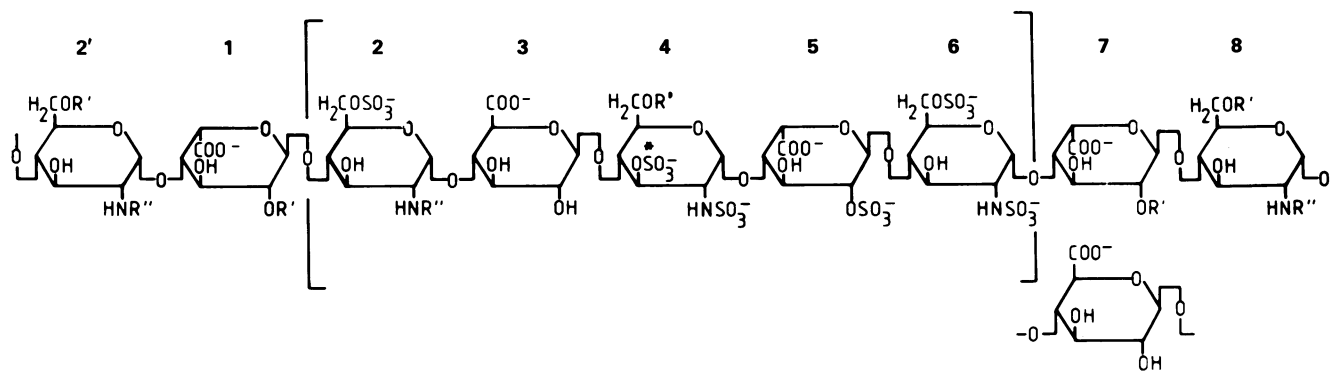


Fig. 1. Structure of the antithrombin-binding region in heparin

The numbering of the monosaccharide units is the same as in previous papers (see Lindahl *et al.*, 1984). The pentasaccharide sequence 2–6 (within brackets) constitutes the actual binding site. Structural variants are indicated by R' (–H or –SO₃[–]), R'' (–SO₃[–] or –COCH₃), or by the glucuronic acid unit 7 drawn below the main sequence. The glucosaminyl 3-*O*-sulphate group (on unit 4) that is unique to the antithrombin-binding region is indicated by an asterisk (*). For additional information see the text.

non-random fashion in a minor proportion of the polysaccharide chains. In the present study we have addressed the same question in a more direct fashion, using the 3-*O*-sulphated glucosamine residue as a specific structural marker for the antithrombin-binding sequence.

EXPERIMENTAL PROCEDURES

Fractionation and degradation of heparin proteoglycan

The methods used to prepare human antithrombin covalently bound to Sepharose 4B, to isolate biosynthetically ³⁵S-labelled heparin proteoglycan from rat skin, to produce heparin chains from proteoglycans by treatment with NaOH and to produce fragments by incubating heparin proteoglycans with rat serum have been described previously (Horner & Young, 1982).

Heparin proteoglycans, chains and fragments were fractionated on an antithrombin–Sepharose column. The gradient elution protocol has been described in detail previously (Horner & Young, 1982). Briefly, the column is first washed with 0.14 M-NaCl, followed by a shallow gradient from 0.14 M- to 0.40 M-NaCl and a steep gradient from 0.40 M- to 3.0 M-NaCl. The formation of reproducible gradients was greatly facilitated by using an LKB Ultrograd gradient mixer rather than the manually controlled equipment used previously. The Ultrograd mixed varying proportions of solutions containing 0.14 M-NaCl and 3.0 M-NaCl, each of which also contained 0.003 M-CaCl₂ and 0.01 M-Hepes and was adjusted with NaOH to pH 7.3 at ambient temperature. One column (10 cm × 2.2 cm) of antithrombin–Sepharose was used throughout, eluted at a flow rate of approx. 17 ml/h. Effluent fractions (3.0 ml) were collected and were analysed for radioactivity by scintillation counting in PCS (Amersham). The column was washed between fractionations by repeating the entire gradient elution sequence.

After an initial fractionation of the proteoglycan starting material, products PG-A and PG-B (see Fig. 3a) were both re-fractionated (Figs. 3b and 3c). To do this the relevant fractions from the initial fractionation were pooled, mixed with water to lower the NaCl concentration to 0.14 M, and pumped into the affinity column at a flow rate of approx. 40 ml/h. The gel was then washed

with 2 column vol. of 0.14 M-NaCl/0.003 M-CaCl₂/0.01 M-Hepes, pH 7.3, before repeating the standard fractionation procedure.

The assignments of column fractions to pools containing heparins with low affinity and high affinity for antithrombin are shown in Fig. 3. Each product was recovered as follows. Water was added to the relevant pooled fractions to lower the NaCl concentration to 0.2 M. These solutions were pumped into columns of DEAE-cellulose (Whatman DE23; 0.9 cm × 15 cm), to which the [³⁵S]heparins bound quantitatively. The columns were washed with 0.2 M-NaCl, then with 1.0 M-NaCl to elute heparin, which was subsequently precipitated with ethanol. This procedure has been described in detail for the recovery of heparin fragments subsequent to the serum depolymerization reaction (Horner & Young, 1982).

The procedures used to recover heparins by precipitation with ethanol, to determine their mass by measuring metachromasia with Azure A and their anticoagulant activities by the calcium–thrombin time method have been described (Horner & Young, 1982).

Compositional analysis of heparin fractions

The methods used to determine the composition of heparin-related saccharides have been described previously (Thunberg *et al.*, 1982b). Briefly, samples (approx. 10 µg of hexuronic acid) were deaminated with HNO₂ at pH 1.5 and the products were ³H-labelled by reduction with NaB³H₄. Labelling was performed in the presence of Tris buffer as described (Thunberg *et al.*, 1982b) or in Na₂CO₃ buffer (Bienkowski & Conrad, 1985); the two procedures gave similar yields of labelled oligosaccharides, with no apparent difference in composition. The labelled oligosaccharides were isolated by gel chromatography on Sephadex G-15 (1 cm × 170 cm column eluted with 0.2 M-NH₄HCO₃) and were then separated into di- and tetra-saccharides (no significant amounts of larger oligosaccharides were observed in the present experiments) on a column (1 cm × 195 cm, eluted with 0.2 M-NH₄HCO₃) of Sephadex G-25 (superfine grade). Labelled disaccharides were resolved by high-performance ion-exchange chromatography on an Aminex A-25 column eluted with a NaCl gradient (Thunberg *et al.*, 1982b), or (more recently, and owing to the

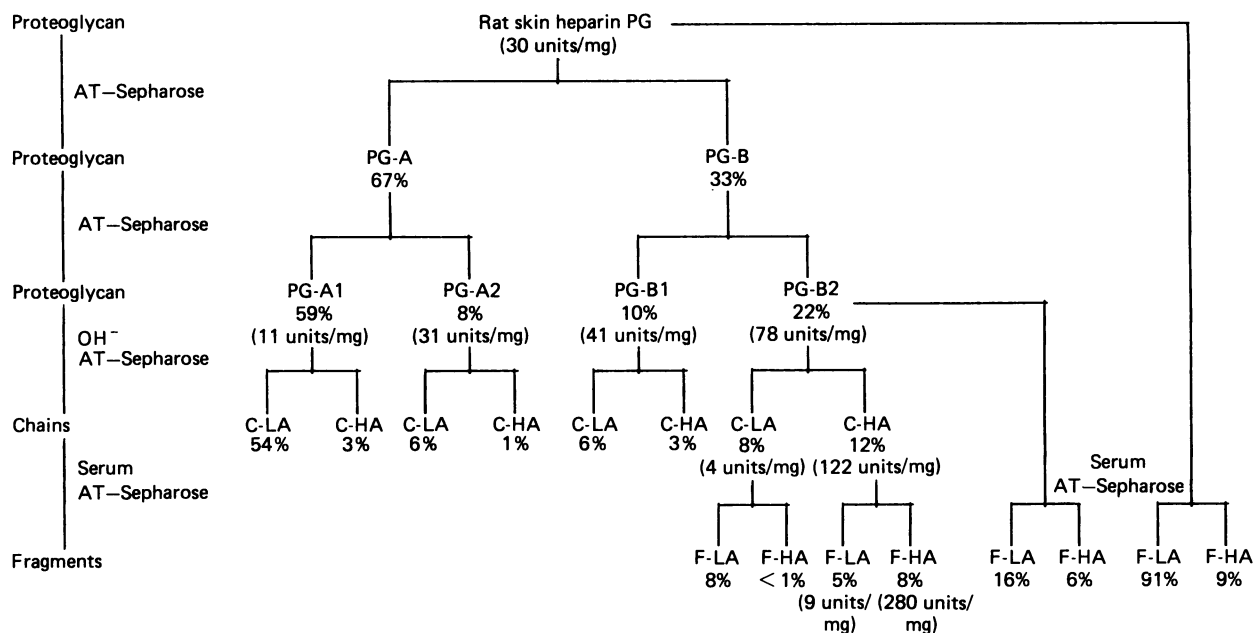


Fig. 2. Fractionation of heparin proteoglycan and its degradation products by affinity chromatography

The rat skin heparin proteoglycan (PG) was separated into four subfractions by repeated affinity chromatography on antithrombin (AT)-Sepharose (Figs. 3a–3c). Alkali (OH^-) treatment of these subfractions yielded single polysaccharide chains (C) which were then separated into fractions with high (HA) or low (LA) affinity for antithrombin (Fig. 3d). Chains and proteoglycan fractions were degraded by incubation with serum, and the resulting polysaccharide fragments (F) were again separated by affinity chromatography (Fig. 3e). The yields of the various fractions are expressed as a percentage of the starting material, assuming 100% recovery in each chromatography step. Chains eluted between the distinct LA and HA fractions (see Fig. 3d), constituting altogether < 10% of the starting material, were excluded. The specific anticoagulant activity (units/mg) is indicated for some of the fractions. For additional information see the text.

corrosive effect of the NaCl on the h.p.l.c. pumps) on a Partisil-10 SAX column using step gradients with increasing concentrations of aqueous KH_2PO_4 (Bienkowski & Conrad, 1985). The latter column was connected to a model Flo-One HS radioactive flow detector (Radiomatic Instruments, Tampa, FL, U.S.A.) using Flo-Scint III (Radiomatic) as scintillation medium. While the order of elution of the various hexuronosyl-anhydromannitol disaccharides differed in the two systems the relative proportions of these components were the same for any given sample tested. The columns were calibrated with reference HexUA- $[\text{^3H}]\text{aMan}_R$ disaccharides, derived from heparin-like polysaccharides and originally separate by procedures involving paper chromatography as the last step (Jacobsson *et al.*, 1979). The two tetrasaccharides, IdUA-GlcNAc(6-OSO₃)-GlcUA-[1- $^3\text{H}]\text{aMan}_R$ -(3-OSO₃) and IdUA-GlcNAc(6-OSO₃)-GlcUA[1- $^3\text{H}]\text{aMan}_R$ (3,6-di-OSO₃), also used for reference purposes, were obtained as described (components *a* and *b*, respectively; Fig. 7a in Thunberg *et al.*, 1982b). The identity of $[\text{^3H}]\text{disaccharides}$ formed on $\text{HNO}_2/\text{NaB}^3\text{H}_4$ treatment of unknown samples was ascertained using ^{35}S -labelled disaccharides, prepared from biosynthetically labelled heparin (Jacobsson *et al.*, 1985) as internal standards.

N-Deacetylation of tetrasaccharides by hydrazinolysis, cleavage of the products by treatment with HNO_2 at pH 3.9 (Thunberg *et al.*, 1982b), and digestion of disaccharides with liver β -D-glucuronidase (type B10; Sigma) (Jacobsson *et al.*, 1979) were performed as described.

RESULTS

The strategy of the present study involved a dissection of the heparin proteoglycan molecule with regard to antithrombin-binding properties, followed by structural characterization of the resulting fractions (proteoglycans, polysaccharide chains, or chain fragments). The overall aim was to define the distribution of the antithrombin-binding regions, as reflected by that of the unique structural determinant, the 3-O-sulphated glucosamine residue.

Fractionation and degradation of heparin proteoglycan

The experimental protocol employed is summarized in Figs. 2 and 3. Affinity chromatography of intact proteoglycan on antithrombin-Sepharose yielded two fractions (denoted PG-A and PG-B in Fig. 3a), of which the high-affinity Fraction B corresponded to approximately one-third of the starting material. Each fraction proved to be heterogeneous on rechromatography; Fraction PG-A yielded a major component (PG-A1) of lower affinity and a minor component (PG-A2) of higher affinity for antithrombin (Fig. 3b), whereas Fraction PG-B separated in the reverse fashion (Fractions PG-B1 and PG-B2 respectively in Fig. 3c). Of the four fractions thus obtained, Fraction PG-A1 had the lowest and Fraction PG-B2 the highest anticoagulant activity whereas Fractions PG-A2 and PG-B1 gave intermediate values (Fig. 2). Each fraction was treated with alkali, under conditions previously found to result in the release

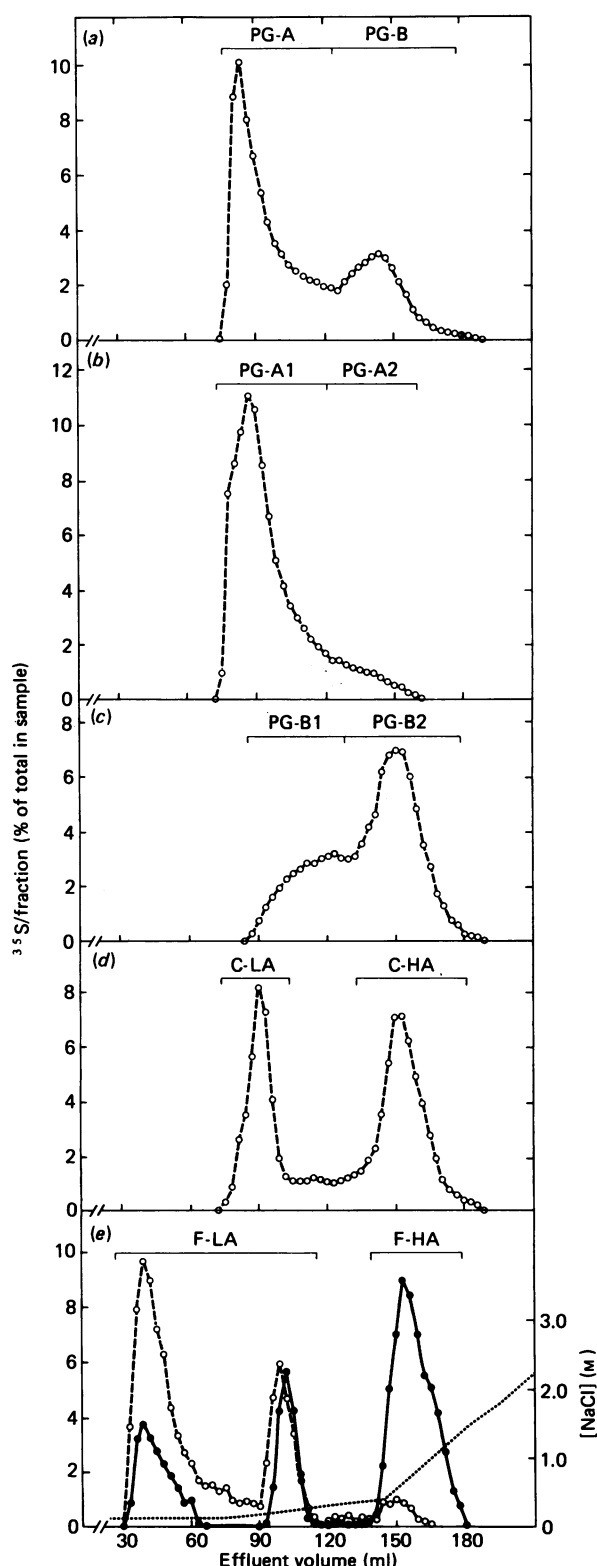


Fig. 3. Affinity chromatography on antithrombin-Sepharose of intact heparin proteoglycans and their degradation products

Chromatography was performed as described in the Experimental procedures section. (a) Rat skin heparin proteoglycan was separated into fractions PG-A and PG-B, which were subjected to rechromatography under the same conditions (panels b and c, respectively). The resulting four subfractions were treated with alkali (0.5 M-NaOH, 4 °C, 72 h), and the released polysaccharide chains were

of single polysaccharide chains (Robinson *et al.*, 1978), and the products were again subjected to chromatography on antithrombin-Sepharose. Distinct fractions of high (HA) and low (LA) affinity were obtained (illustrated in Fig. 3d for the chains derived from proteoglycan Fraction PG-B2), and in addition smaller quantities of incompletely resolved material that was not studied further. The HA proteoglycan fraction PG-B2 yielded HA and LA chains in about equal proportion; the specific anticoagulant activity of the HA chains was higher than that of the parent proteoglycan fraction, whereas the LA chains were essentially devoid of anticoagulant activity (Fig. 2). The major proteoglycan fraction, PG-A1, which accounted for approx. 60% of the starting material, consisted almost exclusively of LA chains, whereas the two fractions of apparent intermediate affinity, PG-A2 and PG-B1, both yielded HA chains in addition to LA chains, albeit in lower proportions than the fraction PG-B2 (Fig. 2).

The free polysaccharide chains derived from the HA proteoglycan fraction PG-B2 were partially depolymerized by incubation with rat serum (see the Experimental procedures section) and the resulting fragments [M_r approximately similar to that of commercially available heparin (Horner & Young, 1982)] were subjected to affinity chromatography. While the LA chains yielded essentially LA fragments only (see definition in the legend to Fig. 3), about 60% of the products formed on fragmentation of the HA chains retained high affinity for antithrombin (Figs. 2 and 3e). In contrast, similar depolymerization of the unfractionated proteoglycan starting material yielded only 9% HA fragments (Fig. 3e). The HA chains derived from the proteoglycan fraction PG-B2 thus accounted for approx. 10% of the mass, but contained more than half of the antithrombin-binding regions of the unfractionated proteoglycan [Fig. 2; this estimation assumes that the antithrombin-binding regions in the proteoglycan and in the isolated HA chains were equally accessible to cleavage by the endoglycosidase (Thunberg *et al.*, 1982a) in serum]. The specific anticoagulation activity of the isolated HA fragments was approx. 9-fold greater than that of the initial

fractionated as illustrated for fraction PG-B2 in (d). Panel (e) shows the chromatograms obtained after digestion of unfractionated proteoglycan (○) and of HA chains from fraction PG-B2 (●) with serum. NaCl concentration (----) was determined by measuring conductivity. This concentration gradient was generated reproducibly in all the above fractionations, using an LKB Ultrograd with a Pharmacia peristaltic pump. The left ordinate on each panel indicates ^{35}S radioactivity, expressing the radioactivity in each fraction as a percentage of the total radioactivity in the sample. In the present report the term 'LA fragments' denotes material that passes essentially straight through the antithrombin-Sepharose column as well as components that are retained on application to the column, but are eluted in the initial shallow part of the NaCl gradient. These fractions, which appear clearly separated on affinity chromatography (Fig. 3e) and were referred to as 'no-affinity' and 'low-affinity' fragments, respectively, in a previously paper (Horner & Young, 1982), differ mainly with regard to molecular size. Neither of the two types of fragments shows any significant anticoagulant activity, nor do they contain any detectable 3-O-sulphated glucosamine residues (results not shown).

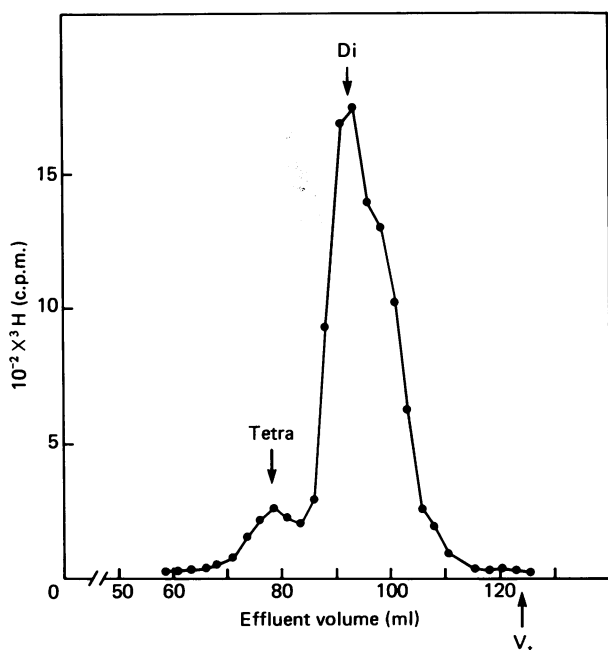


Fig. 4. Gel chromatography of labelled saccharides obtained by $\text{HNO}_2/\text{NaB}^3\text{H}_4$ treatment of heparin fraction

Heparin samples were treated with HNO_2 and the products were reduced with NaB^3H_4 and isolated by passage through a column of Sephadex G-15 (Thunberg *et al.*, 1982b). The labelled saccharides obtained were applied to a column (1 cm \times 195 cm) of Sephadex G-25 (superfine grade), equilibrated with 0.2 M- NH_4HCO_3 , and were then eluted with this salt solution at a rate of approx. 2 ml/h. Effluent fractions were analysed for radioactivity. The arrows indicate peak elution positions of di- and tetra-saccharide reference standards derived from heparin. The elution profile shown represents HA-fragments derived from proteoglycan fraction PG-B2, but cannot be distinguished from the corresponding patterns of other fractions. No labelled components larger than tetrasaccharides were observed.

proteoglycan. While it is clear that anticoagulant activities of proteoglycans and their depolymerization products must be compared with caution, these observations are consistent with the previous proposal (Horner & Young, 1982) that the antithrombin-binding regions are accumulated in a minor proportion of the polysaccharide chains of a heparin proteoglycan preparation.

Structural analysis of heparin fractions

Heparin fractions were degraded with HNO_2 at pH 1.5 and the resulting products were radiolabelled by reduction with NaB^3H_4 . In this process glucosaminidic linkages are cleaved and the corresponding glucosamine residues are converted into terminal 2,5-anhydro-D-[1- ^3H]mannitol units (Shively & Conrad, 1976a,b; Thunberg *et al.*, 1982b). Only *N*-sulphated glucosamine residues are susceptible to deaminative cleavage under these conditions; *N*-acetylated glucosamine units are resistant. Assuming that residues 2 and 2' of the sequence shown in Fig. 1 are *N*-acetylated and *N*-sulphated, respectively, as is the predominant situation in preparations analysed thus far (Lindahl *et al.*, 1984; Atha *et al.*, 1984), the glucosamine unit 4 carrying the unique 3-*O*-sulphate

group would be recovered as a terminal [1- ^3H]anhydro-mannitol residue of a tetrasaccharide corresponding to units 1-4 of the intact sequence. If on the other hand unit 2 is *N*-sulphated, as found for a minor proportion of the antithrombin-binding sequences in pig mucosal heparin (Lindahl *et al.*, 1984), units 3 and 4 would be converted into a GlcUA -[1- ^3H]aMan_R(3-OSO₃) or a GlcUA -[1- ^3H]aMan_R(3,6-di-OSO₃) disaccharide. Gel chromatography of the labelled deamination products (Fig. 4) showed, in addition to a major disaccharide peak, a relatively small proportion of tetrasaccharide (approx. 10% of total incorporated radioactivity). This elution pattern was highly similar for all rat skin heparin subfractions investigated.

Analysis of labelled tetrasaccharide fractions by high-performance ion-exchange chromatography failed to show any significant amounts of material with the elution properties of tetrasaccharides derived from units 1-4 of the antithrombin-binding region (result not shown). Moreover, the tetrasaccharides largely (> 90%) resisted cleavage by HNO_2 (pH 3.9) following hydrazin-

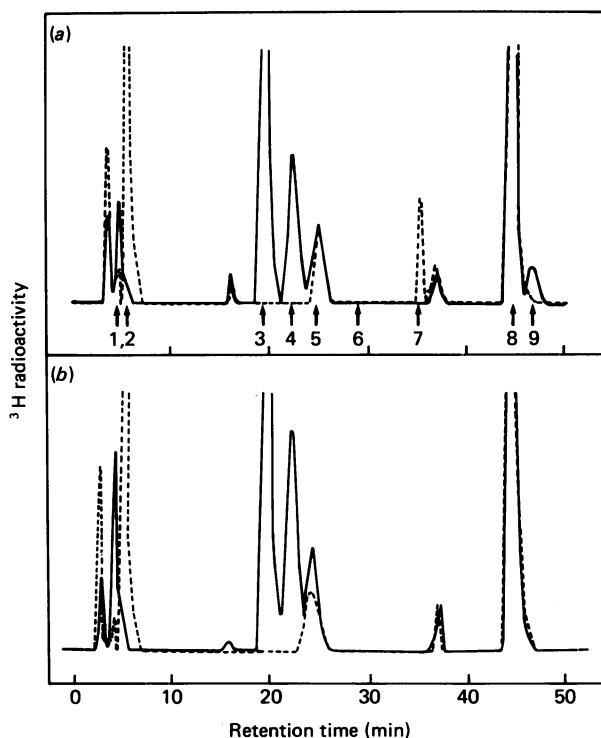


Fig. 5. High-pressure ion-exchange chromatography of labelled disaccharides isolated after $\text{HNO}_2/\text{NaB}^3\text{H}_4$ treatment of (a) HA fragments prepared from proteoglycan fraction PG-B2 and (b) LA chains prepared from proteoglycan fraction PG-A1

Each sample was analysed before (—) and after (----) digestion with bovine liver β -D-glucuronidase. The analyses were performed on a Partisil-10 SAX column eluted first for 30 min with 25 mM- KH_2PO_4 and then with 152 mM- KH_2PO_4 , at a rate of 1 ml/min. The elution positions of reference mono- and di-saccharides are indicated by arrows: 1, HexUA-aMan_R; 2, aMan_R(6-OSO₃); 3, GlcUA-aMan_R(6-OSO₃); 4, IdUA-aMan_R(6-OSO₃); 5, IdUA(2-OSO₃)-aMan_R; 6, GlcUA-aMan_R(3-OSO₃); 7, aMan_R(3,6-di-OSO₃); 8, IdUA(2-OSO₃)-aMan_R(6-OSO₃); 9, GlcUA-aMan_R(3,6-di-OSO₃).

Table 1. Products formed on deamination of heparin fractions

Preparation*	Percentage of disaccharide units† recovered as:				
	GlcUA-aMan _R (6-OSO ₃)	IdUA-aMan _R (6-OSO ₃)	IdUA(2-OSO ₃)-aMan _R	IdUA(2-OSO ₃)-aMan _R (6-OSO ₃)	GlcUA-aMan _R (3,6-di-OSO ₃)
Proteoglycans					
PG-A1	26	14	8.0	52	n.d.‡ (n.d.)§
PG-B2	29	13	6.6	51	1.2 (1.2)
Chains					
PG-A1→C-LA	27	14	6.7	52	n.d. (n.d.)
PG-A2→C-LA	29	10	6.0	53	n.d.
PG-B1→C-LA	27	12	4.8	54	n.d.
PG-A2→C-HA	20	9.7	4.5	63	1.2
PG-B1→C-HA	27	9.7	5.8	54	1.5
PG-B2→C-HA	30	12	5.9	51	2.1 (1.8)
Fragments					
PG-UF→F-LA	15	7.7	4.8	71	n.d.
PG-B2→F-LA	23	12	7.2	54	n.d.
PG-UF→F-HA	22	12	7.8	56	2.3 (2.9)
PG-B2→F-HA	25	11	5.8	55	3.1 (3.4)

* The parent proteoglycan (PG) fraction for each polysaccharide chain (C) or fragment (F) preparation is indicated by arrows. The fragment preparations analysed were all isolated after direct degradation of proteoglycans (UF, unfractionated; PG-B2, see Fig. 2) by digestion with serum. For further information regarding the various fractions, see Fig. 2.

† Calculated after separation of the HexUA-[1-³H]aMan_R disaccharides formed on HNO₂/NaB³H₄ treatment of heparin fractions (see 'Experimental procedures').

‡ N.d., none detected. Under the conditions of the analysis the limit of detection for a single component corresponded to about 0.3% of the total disaccharide contents.

§ Values in parentheses refer to the amounts (expressed as percentage of the total ³H incorporated) of aMan_R(3,6-di-OSO₃) monosaccharide detected after digestion of the unfractionated labelled disaccharides with β-glucuronidase (see Fig. 5).

olysis, but were readily converted into disaccharides by mild acid hydrolysis (25 mM-H₂SO₄, 80 °C for 30 min). These findings suggested the occurrence of so-called 'ring contraction tetrasaccharides', formed during the initial deamination reaction (at pH 1.5) by loss of *N*-sulphate groups and contraction of glucosamine residues to 3-aldehydopentose units, without cleavage of the corresponding glycosidic linkages (Shively & Conrad, 1976b; Thunberg *et al.*, 1982b; Bienkowski & Conrad, 1985). Such tetrasaccharides would not contain any *N*-acetylated glucosamine residues and there was no evidence to suggest that they were preferentially derived from antithrombin-binding regions. Bienkowski & Conrad (1985), working with commercial heparins, have reported that the compositions of ring-contraction tetrasaccharides reflect the overall disaccharide composition of the intact polysaccharide.

High-performance ion-exchange chromatography of the disaccharide fractions showed largely similar elution patterns for all samples investigated (Fig. 5, Table 1). A peak of unretarded, ³H-labelled (unidentified) material was closely followed by a component, probably non-sulphated HexUA-aMan_R disaccharide, that was largely eliminated by digestion of the samples with β-D-glucuronidase prior to chromatography. Of the sulphated disaccharides, IdUA(2-OSO₃)-aMan_R(6-OSO₃) was invariably the predominant component (Table 1), followed by GlcUA-aMan_R(6-OSO₃), IdUA-aMan_R(6-OSO₃), and IdUA(2-OSO₃)-aMan_R, in the order of decreasing abundance. The identity of the disaccharides was confirmed by digestion with β-D-glucuronidase; the commercial preparation employed also contained α-L-iduronidase and thus degraded all species with non-sulphated hexuronic acid moieties (Fig. 5). The relative

amounts of these disaccharides varied somewhat from one heparin fraction to another, with no apparent correlation with the antithrombin-binding properties of the material (Table 1).

Contrary to these major disaccharides which occurred in all samples, a minor component which emerged immediately after IdUA(2-OSO₃)-aMan_R(6-OSO₃) was detected only in some of the chromatograms (Fig. 5). The identity of this component with the disaccharide GlcUA-aMan_R(3,6-di-OSO₃), as indicated by the elution position of the authentic disaccharide standard (see also Lindahl *et al.*, 1984; Bienkowski & Conrad, 1985), was verified by digestion with β-D-glucuronidase, which eliminated the disaccharide and produced a novel labelled compound with the elution properties of aMan_R(3,6-di-OSO₃) (Fig. 5a). Formation of this disulphated monosaccharide was observed only with samples that displayed the putative parent disaccharide (Table 1). The related mono-*O*-sulphated disaccharide, GlcUA-aMan_R(3-OSO₃), was not detected in any of the samples. Current knowledge of heparin structure indicates that deamination products containing a terminal aMan_R(3-OSO₃/3,6-di-OSO₃) residue derive from the antithrombin-binding region (Lindahl *et al.*, 1984). A survey of the various heparin fractions showed that the occurrence of 3,6-di-*O*-sulphated glucosamine residues was strictly correlated with high affinity for antithrombin (Table 1). The disaccharide GlcUA-aMan_R(3,6-di-OSO₃) thus was readily identified after deamination of all HA chain and fragment fractions but was not obtained in detectable amounts from any of the corresponding LA fractions. It was also found in the extreme HA proteoglycan fraction (PG-B2) but not in the extreme LA proteoglycan fraction [PG-A1; the proportion of HA

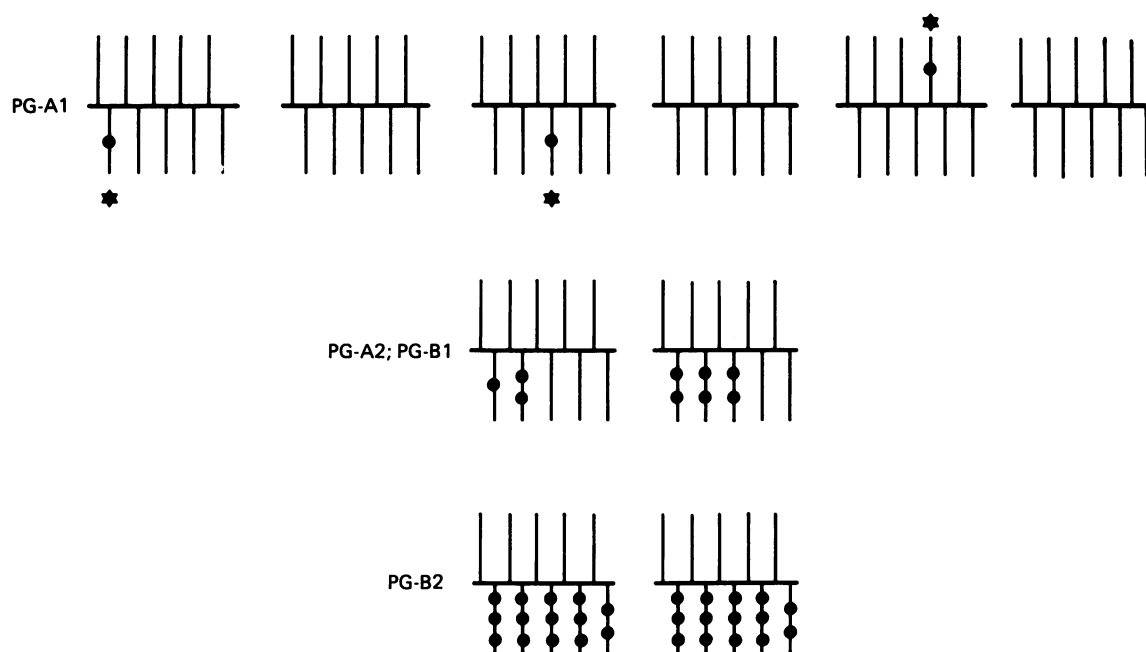


Fig. 6. Schematic display of the distribution of antithrombin-binding regions in heparin proteoglycan molecules

A population of 10 proteoglycan molecules, each with 10 polysaccharide chains, is shown, allowing an approximative quantitative representation of subclasses with different antithrombin-binding properties. The small filled circles on the polysaccharide chains indicate antithrombin-binding regions. The number of such regions per polysaccharide chain was estimated assuming a chain M_r of 80000. The M_r of polysaccharide chains released from a similar preparation of rat skin proteoglycan was previously found to be 60000–100000, by a variety of methods (Robinson *et al.*, 1978). The gel chromatography properties of such chains (peak K_{av} = 0.45 on Sepharose 4B; Fig. 2B in Robinson *et al.*, 1978) were closely mimicked by the single chains described in the present report (result not shown). The HA chains derived from Fraction PG-A1 (marked by asterisks) were not subjected to structural analysis, and the number of antithrombin-binding sites per chain can therefore not be specified. For additional information see the text.

chains in this fraction (Fig. 2) would be too low to permit the detection of GlcUA- α Man_R(3,6-di-OSO₃) disaccharide after deamination]. Moreover, the isolated HA fragments yielded larger amounts of the 3,6-di-*O*-sulphated disaccharide than did the parent HA chains, in accord with the observation that serum digestion converted these chains into a mixture of HA and LA fragments (Fig. 3e, Table 1). In the major fraction of HA chains, derived from the proteoglycan pool PG-B2, the specific marker disaccharide residue accounted for 2.1% of the total disaccharide units. Assuming an average M_r of 80000 for the single polysaccharide chain (see the legend to Fig. 6), and an average M_r of 600 for the constituent disaccharide unit, each chain would carry approximately three (2.8) antithrombin-binding regions.

DISCUSSION

Heparin proteoglycans and their constituent polysaccharide chains were fractionated with regard to affinity for antithrombin and the composition of the products was determined. Based on these results, and on the assumption that the 3-*O*-sulphated glucosamine residue serves as a unique marker for the antithrombin-binding region, the chart shown in Fig. 6 illustrates the distribution of such regions within the overall proteoglycan population. This population is represented by 10 proteoglycan molecules, each containing 10 polysaccharide chains. Essentially three types of molecules may be

visualized. About 60% of the proteoglycans (PG-A1; see also Fig. 2) are practically devoid of any antithrombin-binding sites; it is difficult to judge whether the occasional HA chains formed on alkaline β -elimination actually derive from the major proteoglycan fraction or from contaminating proteoglycans with inherent higher affinity for antithrombin. A second class of proteoglycans is represented by fractions PG-A2 and PG-B1, which together constitute about 20% of the total population. The main portions of these fractions appeared to be intermediate between fraction PG-A1 and fraction PG-B2 with regard to affinity for antithrombin (Fig. 3). These molecules contain a relatively low proportion (15–30%) of HA chains, each chain carrying on the average two antithrombin-binding regions. A major portion (approx. 60%) of the anticoagulant activity of the starting material was recovered in the third subclass of proteoglycan, PG-B2, which also constituted about 20% of the total population. In these macromolecules about half of the chains displayed on the average three antithrombin-binding regions, whereas the remaining chains lacked such regions.

The display of antithrombin-binding sites in Fig. 6 deliberately emphasizes a non-random array of these sites and may be in error in some respects. For instance, we do not know that the binding regions, as indicated, are uniformly distributed between the HA chains of a given proteoglycan molecule, nor do we have any information regarding the distribution of HA and LA

chains within the individual proteoglycan molecules. However, regardless of this bias it is evident that the antithrombin-binding regions are distributed in a highly non-random fashion. Thus, 20% of the proteoglycan molecules, and 10% of the polysaccharide chains, contained about two-thirds of the total binding sites, whereas most of the proteoglycan molecules essentially lacked this structure. Conversely, in the proteoglycan fraction with the highest content of binding region (PG-B2) these regions occurred in only half of the chains. These conclusions are in general agreement with previous observations based essentially on determinations of anticoagulant activity (Horner & Young, 1982).

The non-random distribution of antithrombin-binding regions in the heparin proteoglycan raises some questions regarding the biosynthesis of this macromolecule. The complex structure of the completed heparin chain is acquired through polymer-modification reactions (*N*-deacetylation and *N*-sulphation of D-glucosamine residues, C-5-epimerization of D-glucuronosyl to L-iduronosyl units, and *O*-sulphation at various positions) which are catalysed by a series of membrane-bound enzymes, in a stepwise, highly organized, fashion (Jacobsson *et al.*, 1984; Lindahl *et al.*, 1986). It was previously hypothesized that the retention of the *N*-acetyl group at unit 2 in the antithrombin-binding region (see Fig. 1) might serve as a biosynthetic signal promoting the assembly of the functional binding site (Lindahl *et al.*, 1984), such that the final location of this region would be determined, or strongly influenced, by regulation of the initial *N*-deacetylation step. This notion may still be valid for heparins with antithrombin-binding regions that are predominantly *N*-acetylated at unit 2, such as pig mucosal heparin. However, it would not seem to apply to rat skin heparin, which is apparently *N*-sulphated rather than *N*-acetylated at unit 2. In search for alternative mechanisms of regulation one may consider factors such as the location of key enzymes in the endoplasmic membranes (probably the Golgi system) of the mast cell. Indeed, a restricted distribution of 3-*O*-sulphotransferase molecules in the membrane might explain the non-random distribution of antithrombin-binding regions in the heparin proteoglycan (see Lindahl *et al.*, 1986).

This work was supported by: Grant 2309 from the Swedish Medical Research Council and Grant 600/83D98:2 from the Swedish Council for Forestry and Agricultural Research; the Swedish National Board for Technical Development; the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences; KabiVitrum AB, Stockholm, Sweden; the Heart and Stroke Foundation of Ontario.

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